ISOLATION AND PHARMACOLOGICAL CHARACTERIZATION OF VERNOLEPIN

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ABSTRACT.—Vernolepin, a sesquiterpene lactone, was isolated from the dried fruit of *Vernonia amygdalina* Del. The different steps used during the extraction were: continuous extraction with chloroform, partition of the chloroform-extract between pertroleum ether and 10% aqueous methanol, column chromatography of the methanol extract, isolation of the active fractions by pharmacological and chemical characterization. Vernolepine was obtained as colorless prisms and identified by melting point, uv, ir, ^{1}H nmr, optical rotation, and mass spectrometry. The total content of the dried fruit was 0.09% vernolepin. The first pharmacological characterization of vernolepin revealed: (1) a competitive antagonism against histamine in guinea pig ileum ($pA_2 = 5.61$; 15 min incubation); (2) a biphasic enhancement/inhibition of coaxial stimulation of guinea pig ileum; (3) an antiaggregating and disaggregating activity against rabbit platelet aggregation induced by arachidonic acid (1×10^{-4} g/ml; 3.3×10^{-4}M) or ADP (4×10^{-6} g/ml; 1×10^{-5}M) without inhibition of cyclo-oxygenase or lipoxygenase. All these reactions were time dependent and occurred at concentrations of 5×10^{-6} to 1×10^{-5} g/ml vernolepin (1.8 to 3.5×10^{-5}M).

An alcoholic extract of *Vernonia amygdalina* Del. (Compositae) had been shown to possess significant activity in our pharmacological screening program. Consequently, a systematic study aimed at the isolation of the pharmacologically active principle(s) was undertaken. In Africa, *Vernonia amygdalina* is used for variable indications depending on the part of the plant and geographical localization. Root, stem, bark, fruit, leaves and twigs are against such conditions as schistosomiasis, fever, gastrointestinal upset and even as a foodstuff. West African women eat the leaves in the belief that it renders them sexually more attractive (1).

The leaves of *Vernonia amygdalina* have already been documented to yield vernodalin and vernomygdin. These sesquiterpene lactones have been shown to have significant inhibitory activity *in vitro* against cells derived from human carcinoma of the nasopharynx carried in tissue culture (2). Two similar compounds, *viz.* vernolepin and vernomenin, have been isolated from the dried ground leaves of *Vernonia hymenolepis* A. Rich; these compounds also show tumor inhibitory activity in the same tissue culture model (3, 4). Other sesquiterpene lactones isolated from different *Vernonia* species are vernolide (5), hydroxy-vernolide (6), and pectorolide (7). This paper deals with the isolation and first pharmacological characterization of vernolepin from the dried fruit of *Vernonia amygdalina*.

EXPERIMENTAL

PREPARATION OF PLANT EXTRACTS.—Routinely, 5 g of dried ground material were macerated in 150 ml of distilled ethanol (80%). After stirring at room temperature for 3 hours, the macerated plant suspension was filtered through a glass pore filter (n° 3), and the marc was washed with ethanol (80%). The filtrate and washing ethanol were combined and concentrated to near dryness under reduced pressure at a temperature not in excess of 40°. The residue was diluted to 10 ml with distilled water and stored at $-30°$ until tested. Aliquots of 1 ml of these extracts were used in single-dose-response pharmacological screening tests.

ISOLATION OF VERNOLEPIN.—A slightly modified procedure, described by Kupchan *et al.* (4), was used (figure 1). About 400 g of dried ground fruit of *Vernonia amygdalina* were continuously extracted for 2 x 16 hours with 2 liters chloroform^1 in a Soxhlet reflux apparatus. The resulting extract was concentrated in a rotating evaporator under reduced pressure to yield a thick oil (B, ±30 g). Fraction A (±40 g) was obtained by continuous extraction of the marc for 16 hours with distilled methanol, since it showed no activity, it was discarded. Fraction B, when partitioned between 300 ml of petroleum ether (40°-60°) and 300 ml of 10% aqueous methanol, yielded after evaporation 10% aqueous methanol solubles (12.3 g). A

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^{1}When not specified, reagents were of analytical grade (Merck).

portion of these solubles (4.0 g) dissolved in chloroform (10 ml) was chromatographed on a silica gel column (length 80 cm, internal diameter 4 cm, 400 g silica gel 60 Merck, 70–230 mesh ASTM), packed in chloroform. The column was developed with a gradient of chloroform changing to chloroform-acetone and finally to acetone.

Fractions of 20 ml were collected[2], and monitoring was done by thin layer chromatography analysis of every third fraction (10 μ l aliquots, DL Plastikfolien kiezelgel 60 F 254, 20 x 20 cm, schichtdicke 0.2 mm, Merck) in methanol-acetone-chloroform (5:15:80). Spots were located by uv illumination and spraying with 3% Ce $(SO_4)_2$ in 3N H_2SO_4 followed by heating at 110°. The fractions which showed identical patterns were combined and evaporated to dryness. The residue was triturated with 20 ml of methanol, and aliquots of 2 ml were again evaporated to dryness, then dissolved in 10–25% DMSO, and finally pharmacologically tested by coaxial stimulation of the guinea-pig ileum.

The pharmacologically most active fraction was further chromatographed on a preparative semi-high pressure liquid chromatography Merck Lobar type B column of 31 cm length and 2.5 cm internal diameter, packed with Lichroprep Si60 (40–63 μ diameter) in chloroform. The column was developed with a gradient of chloroform changing to chloroform-acetone and finally to acetone at a flow rate of 1.1 ml/min and a pressure of about 10 kg/cm² [3]. Fractions were collected and monitored as described earlier. The pharmacologically most active fraction was evaporated to dryness, and triturated with anhydrous ether yielding an amorphous solid, which, when crystallized from chloroform-petroleum-ether, gave colorless prisms of vernolepin. This material was chemically characterized[4].
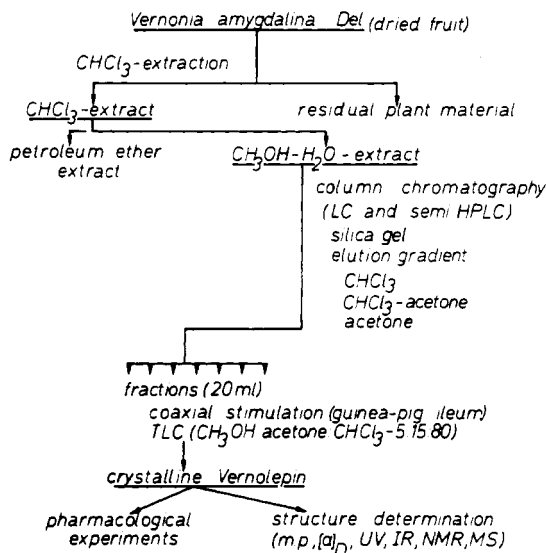
EXTRACTION of VERNOLEPIN

FIG. 1. Extraction of vernolepin: the slightly modified procedure according to Kupchan et al. (4) was used.

SINGLE-DOSE SCREENING.—Guinea pigs (300–500 g) were killed by a blow on the head and exsanguinated; sections of their ileum were prepared and mounted in an isolated organ bath (40 ml) bubbled with a 95% O_2–5% CO_2 mixture at 37°. Tyrode was used as the bathing fluid with concentrations in g/l (mM): 0.2 KCl (2.7), 0.13 $MgSO_4.7H_2O$ (0.53), 0.065 $NaH_2PO.2H_2O$ (0.42), 0.27 $CaCl_2.2H_2O$ (1.84), 8.0 NaCl (136), 1.0 $NaHCO_3$ (11.9), 1.0 glucose (5.55) (p.a. Merck). Contractions were measured auxotonically (8); 0.75 g tension was applied[5]. The technique was that of single doses as introduced by Magnus (9). Control contractions were obtained with acetylcholine (acetylcholine Cl, Merck) or histamine (histamine.diCl Pharma-

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[2]A LKB 7000 Ultrarac fraction collector was used.

[3]Pressure was obtained by using an Orlita DMP–AE–10.4 pump.

[4]The melting point was determined on a Büchi SMP–20 apparatus and was corrected. The optical rotation was determined with a Perkin-Elmer 241–MC polarimeter. The ultraviolet spectrum was obtained on a Beckman Acta C III UV-instrument and the infrared spectrum was recorded on a Beckman Acculab TM 4 instrument. The ¹H-nmr spectrum was recorded on a Jeol JNM–PFT 100 spectrometer operating at 100 MHz. Chemical shifts (δ) are given in ppm values from TMS as internal standard. The mass spectrum was determined on a Jeol 01 SG II double focusing instrument coupled to a JEC–6 spectrum computer and operating at 70 eV.

[5]Harvard smooth muscle transducers (model 386) were used. Contractions were recorded on a Bryans 28000 two-channel recorder.

chemic) 3 x 10⁻⁸ g/ml[6]. A plant extract was added to the bath and incubated for 5 min before a standard dose of agonist was added. Agonist contractions before and after the extract were compared.

CUMULATIVE DOSE-RESPONSE EXPERIMENTS.—The same preparation and experimental conditions were used for cumulative dose-response curves with acetylcholine, histamine and BaCl₂. Concentrations of 1 x 10⁻⁹ g/ml to 1 x 10⁻⁴ g/ml of acetylcholine and histamine and of 1 x 10⁻⁶ to 3 x 10⁻³ g/ml of BaCl₂ were applied in a cumulative way according to Van Rossum (10). Contractions of the same strip before and after incubations (3 to 60 min) with vernolepin 5 x 10⁻⁶ and 1 x 10⁻⁵ g/ml dissolved in 5 and 10% DMSO, respectively, were compared.
For the experiments with BaCl₂, Krebs solution without sulfate was used (concentrations were the same as for normal Krebs but MgSO₄.7H₂O was replaced by an equimolar amount of MgCl₂).

COAXIAL STIMULATION.—A segment (6–7 cm) of guinea pig ileum was mounted for electrical stimulation, as described by Paton (11), in a 40-ml organ bath containing Krebs solution, bubbled with 95% O₂ and 5% CO₂ and maintained at 37°. The Krebs solution had the following composition in g/liter (mM): 6.9 NaCl (118), 0.35 KCl (4.7), 0.55 CaCl₂.2H₂O (2.5), 0.16 KH₂PO₄ (1.2), 0.29 MgSO₂.7H₂O (1.17), 2.0 glucose (11.1), 2.1 NaHCO₃ (25.0). Contractions were measured auxotonically, with a load of 1 g [5, 7]. Column chromatography eluates and vernolepin were tested on this model. Before and after the stimulation "run", exogenous acetylcholine (5 x 10⁻⁷ g/ml) was applied, and the subsequent contractions were compared.

PLATELET AGGREGATION.—Rabbits weighing approximately 1.5-2.5 kg were anesthetized with pentobarbital sodium (60 mg/ml, Nembutal® Abbott). The dose was 30 mg/kg and the solution was diluted 1/1 with 0.9% NaCl in order to avoid hemolysis. Heart puncture was done, and blood (9 ml portions) was collected in plastic tubes with 1 ml 3.8% trisodium citrate (Merck). Tubes were gently mixed and centrifuged at 1500 rpm during 10 min to obtain platelet-rich plasma[8]. Platelet-poor plasma was prepared by centrifugation at 3000 rpm. Platelet aggregation was performed in an aggregometer[9] according to the turbidimetric method of Born (12). Aliquots of 0.2 ml of platelet-rich plasma were used. The aggregations were initiated by addition of arachidonic acid (Sigma, 1 x 10⁻⁴ g/ml; 3.3 x 10⁻⁶M final concentrations) or ADP (Sigma, 4 x 10⁻⁶ g/ml; 1 x 10⁻⁵M final concentration) and followed for at least 10 min[10]. Final concentrations of vernolepin 1 x 10⁻⁵ g/ml, were compared with 0.1% DMSO. Incubation times of DMSO and vernolepin were 1, 5 and 10 min before the aggregation was initiated.

INCUBATION OF WASHED RABBIT PLATELETS WITH ARACHIDONIC ACID.—A suspension of washed rabbit platelets was prepared according to Hamberg *et al.* (13). Rabbit blood (10 ml) was collected as described for the aggregation studies and gently mixed with 0.75 ml 77 mM disodium EDTA (UCB). As much as 50–90 ml was collected from each animal. Platelet-rich plasma was prepared, the platelets were isolated (2500 rpm, 10 min) and resuspended in buffer (0.15 M NaCl: 0.15 M Tris pH 7.5: 77 mM EDTA–90:8:2 by volume), 1 ml for each platelet pellet obtained from 10 ml of blood. Platelets were gently mixed and centrifuged (10 min 2500 rpm). The pellet was resuspended in Ca⁺⁺–free Krebs solution saturated with 95% O₂ and 5% CO₂ containing g/liter (mM) 0.7 KCl (9.4); 0.6 MgSO₄7H₂O (2.4); 0.32 KH₂PO₄ (2.4); 13.8 NaCl (237.9); 2.1 NaHCO₃ (25.0); 1 glucose (5.5). A suspension of approximately 500,000 platelets/μl was obtained. Platelets (1 ml suspension) were subsequently incubated with 30 μg unlabeled (Sigma) and 100.000 dpm C¹⁴ labeled (NEN-chemicals) arachidonic acid for 30 min in a shaking waterbath at 37°C. Reactions were stopped by addition of 30 μl 2M citric acid and 10 μl prostaglandin cocktail (E₂; F₂α, thromboxane B₂, arachidonic acid 1 μg/μl for all the components[11]. The incubation mixture was extracted twice with 2 ml of ethylacetate by mechanical mixing and centrifugation at 3000 rpm for 5 min. Combined ethylacetate was evaporated under N₂ at 37° and the residue dissolved in 50 μl chloroform-methanol (2:1), spotted on a tlc plate (Silicagel 60F 254, 0.25 mm Merck). The plate was developed with chloroform-methanol-acetic acid-water (90:6:1:0.6) according to Nugteren and Hazelhof (14), and scanned [12]. After scanning, 10% phosphomolybdic acid in ethanol was sprayed over the plate and the authentic prostaglandins visualized by heating (5 min at 105°). The different zones were removed from the plate. After addition of 10 ml Instagel (Packard), radioactivity (dpm) was measured[13].

RESULTS AND DISCUSSION

ISOLATION AND IDENTIFICATION OF VERNOLEPIN.—The pharmacologically most active fraction was eluted during the preparative semi-high pressure liquid

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[6]Unless stated otherwise, all concentrations are expressed as final bath concentrations of the base.
[7]The parameters for electrical coaxial stimulation (JSI Student Stimulator, Janssen Scientific Instruments) were 40 mA, 1 msec duration and 0.1 Hz.
[8]MSE centrifuge low speed device.
[9]A Payton dual-channel aggregometer.
[10]A Kipp & Zonen BD 41 two channel recorder was used.
[11]Prostaglandins were kindly supplied by Dr. J. Pike, Upjohn.
[12]A Berthold LB 2760 scanner was used.
[13]A Packard 2650 liquid scintillation counter was used; the external standard ratio for quench correction was used. Results were expressed as a percentage of the total radioactivity recovered from the plate.

chromatography with a mixture of chloroform-acetone (95:5). Evaporation to dryness, trituration of the residue, and crystallization of the resulting amorphous solid yielded 0.120 g (0.09% calculated on dried fruit of *Vernonia amygdalina*) vernolepin as colorless prisms, mp 179–180°; $[\alpha]^{20}D+71°$ (c 1.1, acetone); tlc Rf 0.38; uv λ max (CH$_3$OH) 220 nm (end absorption, log ϵ 4.29); ir ν max (KBr) 3600–3150 cm^{-1}, 3580, 1100 (secondary-OH), 3080, 2975 (unsaturated-CH$_2$), 1790 (–C=O, γ-lactone), 1740 (–C=O, δ-lactone), 1625 (–C=C–, terminal methylene and vinyl), 1420, 990 (terminal methylene), 940 (vinyl); ^{1}H nmr (100 MHz, CDCl$_3$), δ 6.70 (d, 1H, $J=1$ Hz, H–15), 6.21 (d, 1H, $J=3$ Hz, H–13), 6.02 (d, 1H, $J=3$ Hz, H–13), 5.91 (d, 1H, $J=1$ Hz, H–15), 5.41 (complex multiplet, 3H, H–1 and H–2), 4.23 (complex multiplet, 4H, H–6, H–8 and H–14), 2.95 (m, H–5), 2.66 (tt, 1H, $J=3$ Hz, 5 Hz, 10 Hz, H–7), 2.13 (s, 1H, –OH), 1.80 (m, 2H, H–9), ms (70 eV), m/z; %, 276 (M$^+$.; 14.5), 261 (M.$^+$–15; 3.6), 259 (M.$^+$–17; 9.1). Comparison of these results with those reported in the literature (3, 4) indicated the compound to be vernolepin (figure 2).
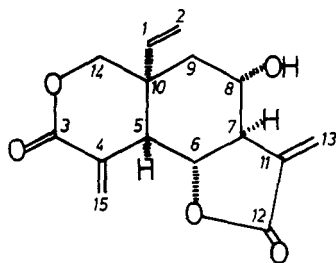
Vernolepin

FIG. 2. Structural formula of vernolepin C$_{15}$H$_{16}$O$_5$.

SINGLE-DOSE SCREENING.—Crude ethanol extracts of *Vernonia amygdalina* fruit strongly inhibited histamine- and acetylcholine-induced contractions after 5 min preincubation. Inhibition was 100% for both agonists (n=2). While the anticholinergic activity disappeared completely after two washings of perfusate, the antihistaminic activity was irreversible (n=2).

CUMULATIVE DOSE-RESPONSE EXPERIMENTS.—Vernolepin showed a time-dependent inhibitory activity on cumulative doses of acetylcholine and histamine. For acetylcholine there was no inhibitory activity with 5 x 10^{-6} g/ml (n=3) and 1 x 10^{-5} g/ml (n=3) vernolepin when the incubation time was less than 15 min. After 30 and 50 min incubation with 5 x 10^{-6} g/ml (n=3), responses to acetylcholine were depressed to values lower than 40% of the maximum and with 1 x 10^{-5} g/ml (n=2) to less than 20%. Cumulative dose-response curves with histamine after 15-min incubation with 1 x 10^{-5} g/ml vernolepin, showed a parallel displacement to the right, while the maximal response could still be reached at the end of the curves (Figure 3). The pA$_2$=5.61, calculated according to the method of Van Rossum (1963) (n=2) (10). Incubation with vernolepin 1 x 10^{-5} g/ml and 5 x 10^{-6} g/ml for more than 30 min, showed a gradual decrease of the maximal response, but it still remained more than 50% (n=3). For acetylcholine as well as for histamine, the inhibiting activity of vernolepin was not removed by washing; on the contrary, the effect was accentuated after washing. There was no inhibition of cumulative doses of BaCl$_2$ when vernolepin 1 x 10^{-5} g/ml was preincubated for 15 min (n=3). Inhibition towards BaCl$_2$ became apparent, even with 5 x 10^{-6} g/ml vernolepin (n=2), after more than 30 min.

COAXIAL STIMULATION.—Both eluates and vernolepin showed inhibitory activity on the coaxially stimulated guinea pig ileum. Eluates of the first silica

VERNOLEPIN-HISTAMINE
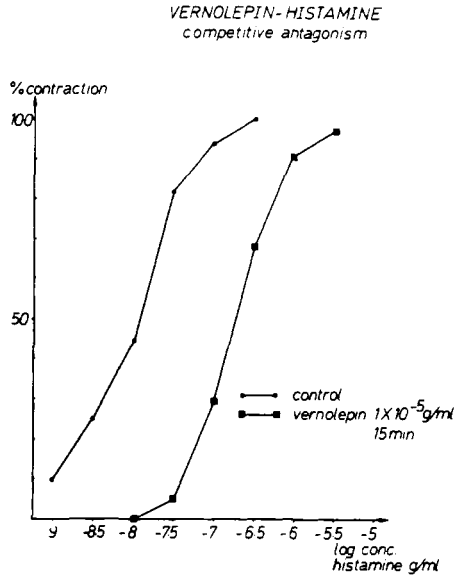competitive antagonism

FIG. 3. Cumulative dose-response experiment. Histamine was used as agonist, vernolepin as antagonist. The control curve and the curve after 15 min vernolepin 1 x 10^{-5} g/ml incubation are the mean values of 2 experiments.

gel column chromatography were tested in an equivalent of 5, 1, 0.5, 0.2 and 0.1 g of dried plant material.   There was a dose- and time-dependent depression of the contractions elicited by coaxial stimulation (figure 4).   There was no inhibition

INFLUENCE of COLUMN ELUATES on
COAXIAL STIMULATION of GUINEA-PIG ILEUM
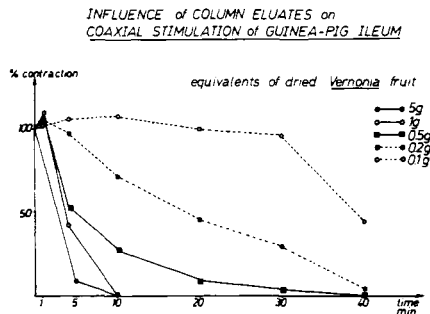
FIG. 4. Eluates obtained by column chromatography were tested on coaxially stimulated guinea-pig ileum. Each curve is the result of 1 experiment. 100% = contraction on time 0.

by the same volume of 10% DMSO.   Vernolepin itself (1 x 10^{-5} g/ml) gave also a clearcut inhibition of the contractions during coaxial stimulation.   After 20 to 40 min, this inhibition was significant ($P < 0.005$ Wilcoxon).   A slight, but significant, enhancement was seen after 3- and 5-min incubation ($P < 0.05$ Wilcoxon, n = 6; figures 5 and 6).

PLATELET AGGREGATION.—The activity of vernolepin on platelet aggregation was of two kinds: anti-aggregating and disaggregating.   The activity was time
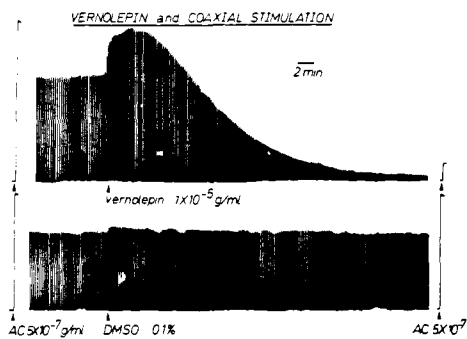
FIG. 5. Time dependent activity of ver-
nolepin 1 x 10^{-5} g/ml vs. DMSO
0.1% on coaxially stimulated guinea-
pig ileum. AC=5 x 10^{-7} g/ml
exogenous acetylcholine without
electrical stimulation.
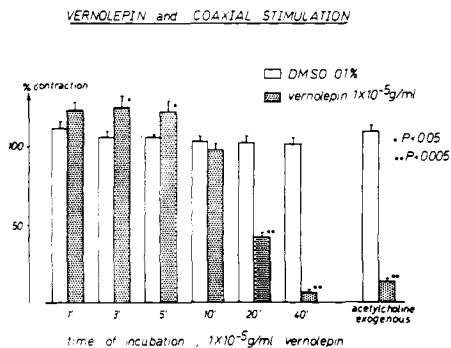
FIG. 6. Time dependent activity of ver-
nolepin vs. DMSO on coaxially
stimulated guinea-pig ileum. P:
Wilcoxon test. Acetylcholine exo-
genous: 5 x 10^{-7} g/ml without co-
axial stimulation. n=6.

dependent, as can be seen from figure 7. The aggregations were always compared
with simultaneous controls which consisted of the vehicle (0.1% DMSO final
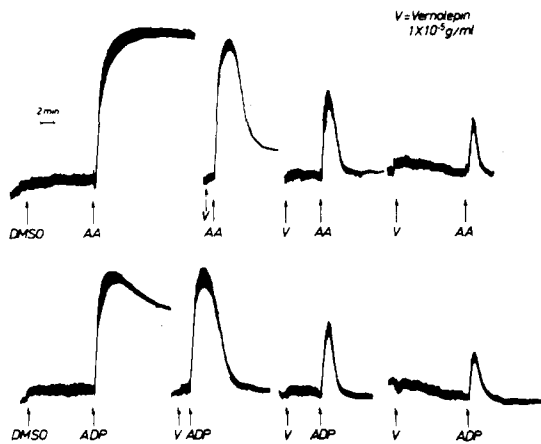concentration) preincubated for the same period. The results are summarized

FIG. 7. Inhibitory action of vernolepin 1 x 10^{-5} g/ml
on rabbit platelet aggregation. DMSO=
dimethylsulfoxyde 0.1%; AA=arachidonic
acid 1 x 10^{-4} g/ml (3.3 x 10^{-4}M); ADP=
adenosinediphosphate 4 x 10^{-6} g/ml (1 x
10^{-5}M).

in figure 8. Significant inhibition of maximum platelet aggregation occurred
after 5- and 10-min preincubations with 1 x 10^{-5} g/ml vernolepin. The residual
aggregation, 10 min after addition of ADP or arachidonic acid, was taken as an
indication of its disaggregating activity. In all cases (1-, 5- and 10-min preincuba-
tions) there was a highly significant disaggregating activity, which meant that
in the presence of vernolepin the aggregation pattern generally returned to the
baseline within 10 min. Also for the final concentration of 5 x 10^{-6} g/ml the
same effects were seen, but it took longer to obtain the same quantitative results
(n=7). Vernolepin given after arachidonic acid or ADP was able to abolish
partially or totally an aggregation already completed (n=3).
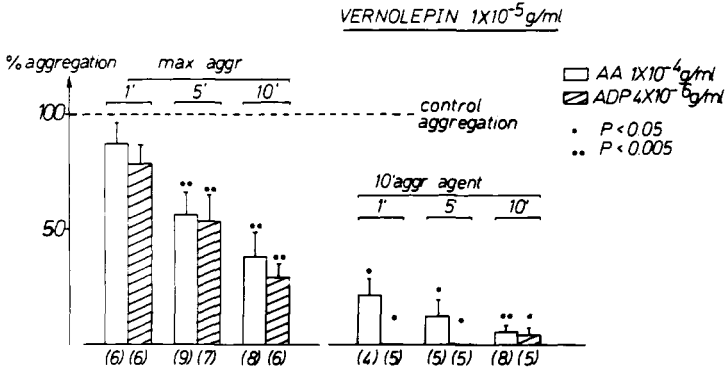
VERNOLEPIN 1X10⁻⁵g/ml

FIG. 8. Antiaggregating and disaggregating activity of vernolepin
against rabbit platelet aggregation.   P: Wilcoxon test. max.
aggr. = maximal aggregation obtained after vernolepin 1 x
10^{-5} g/ml incubations: 10' aggr. agent = residual aggregation
10 min after AA or ADP; control aggregation = aggregation
simultaneously obtained after DMSO 0.1%; number of experi-
ments given at the bottom.

INCUBATION OF WASHED PLATELETS WITH ARACHIDONIC ACID.—Incubation of
washed platelets with radioactive arachidonic acid revealed no differences with
regard to platelet aggregation.   There was a slight increase of biosynthesis for
all the cyclo-oxygenase products (Prostaglandin $F_{2\alpha}$, -E_2, thromboxane B_2, 12-
hydroxyheptadecatrienoic acid) but not for the lipoxygenase product 12-hydroxy-
eicosatetraenoic acid, when incubated with a final concentration of 1 x 10^{-5} g/ml
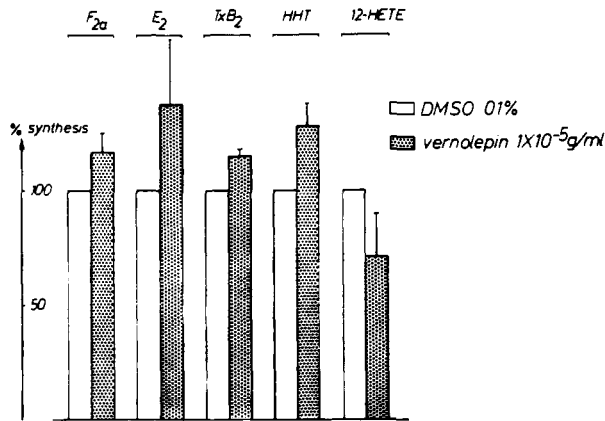vernolepin (n = 4, figure 9).

FIG. 9. Incubation of washed rabbit platelets with
arachidonic acid. Influence of vernolepin 1 x
10^{-5} g/ml *vs.* DMSO 0.1% on biosynthesis of
prostaglandin $F_2$$_\alpha$ ($F_{2\alpha}$), prostaglandin E_2 (E_2),
thromboxane B_2 (TxB_2) 12-hydroxyheptade-
catrienoic acid (HHT), 12-hydroxyeicosatetra-
enoic acid (12–HETE). Each column repre-
sents the mean ± SEM of 4 experiments.

GENERAL DISCUSSION

Several methods for screening of plant extracts have been discussed in literature.
Phytochemical screening (15) or a Hyppocratic and pharmacodynamic screening
(16, 17) might be used.   As already mentioned by Thorp (18), a close cooperation
between pharmacognosists and pharmacologists is necessary.

We chose *in vitro* test models which allowed the testing of extracts. Using guinea-pig ileum, we documented some specific antihistaminic and some non-specific anticholinergic properties for vernolepin. We were also able to evaluate the inhibitory effects of vernolepin on neurotransmission using the coaxial stimulation method of Paton (19). The inhibitory action of vernolepin is most probably due to postsynaptic mechanisms since contractions produced by exogenous acetylcholine were also blocked. An important fact is the time-dependence of the actions. During the first 5 minutes, vernolepin (1 x 10^{-5} g/ml) gave a significant enhancement of contraction. Vernolepin can enhance contraction on several levels: (1) blockade of the cholinesterase, an eserine-like effect (20); (2) enhancement of presynaptic acetylcholine release (21); (3) release of non-cholinergic contractile agents during stimulation (22, 23, 24); or (4) postsynaptic enhancement of the sensitivity to acetylcholine (25). Because no baseline enhancement was observed, one can omit the first hypothesis.

Preliminary results show some potentiation of exogenous acetylcholine contractions of the guinea-pig ileum when vernolepine 1 x 10^{-5} g/ml was incubated during 3 min. Whether or not other mechanisms are involved in this rapid-onset effect has yet to be investigated. The activity of vernolepin against exogenous agonists was also time dependent. For 15-min incubations, cell integrity was maintained according to the $BaCl_2$ experiments. The antihistamine property is more specific than the anticholinergic one since a parallel displacement to the right of the histamine dose-response curve with 15 min incubation occurs without depressing the maximal effect.

During the first screening of crude extracts, antihistaminic activity could not be washed out in contrast with the anticholinergic activity. Cumulative dose-response experiments with vernolepin revealed irreversibility for both antihistaminic and anticholinergic activity. On the first view there seems to be a discrepancy which, however, can be explained: (1) crude extracts were made in ethanol, and extracted components different from vernolepin could influence the activity; (2) as the total content of vernolepin in *Vernonia* fruit is about 0.09%, true vernolepin activity could also be masked; (3) the crude extract was made with ethanol, while vernolepin was extracted with chloroform; (4) it is very difficult to compare directly different techniques as single-dose screening of a crude extract with a cumulative dose-response experiment of a pure compound; (5) for a single-screening a 5-min preincubation was used, while preincubation times were longer for cumulative dose-response experiments.

The quantitative influence on the aggregation patterns was also time dependent. Unfortunately, no specific inhibition of TxA_2-isomerase was detected during platelet incubations. These findings are in conformity with the fact that ADP-aggregations also were blocked and reversed. ADP-aggregations are independent from arachidonic acid and TxA_2 (26, 27, 28).

The exact mechanisms of the antiaggregating and disaggregating activity need to be elucidated. The incubations with washed platelets revealed no deactivation of cyclo-oxygenase, different prostaglandin isomerases and lipoxygenase by 30-min incubations with vernolepin 1 x 10^{-5} g/ml. The cytotoxic activity of vernolepin published by Kupchan *et al.* (4) somewhat overshadows our results. Although no direct comparison can be made between his and our experimental circumstances, we must always be aware of the "alkylating functions" of vernolepin. Therefore, besides further pharmacological evaluation of this substance, its toxicity and therapeutic index should be determined. Such experiments will point out whether vernolepin or some of its derivatives offer interesting therapeutic perspectives, relative to drugs now available.

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